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NADH growth rate evaluation in different rat brain regions by fluorescence spectroscopy

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ABSTRACT

NADH is one of the central signalling molecules that serve as a substrate for many vital processes, in particular, is a donor for the electron transport chain in mitochondria. At the same time, mitochondrial activity and the intensity of metabolic processes in the tissues of different brain regions drastically differ. This work aimed to compare the NAD(P)H production rate in the tissues of various brain regions (cortex, cerebellum, hippocampus, brainstem) assessed by the parameters of dynamics of its autofluorescence. We studied acute slices of the rat brain of the corresponding regions. The intensity of NAD(P)H autofluorescence and its rate of production was determined to assess the activity of mitochondria. Whereafter the slices were analysed by histological examination. It has been observed that mitochondrial activity in hippocampal tissues is significantly higher than in other brain regions, which may be associated with more complex cognitive functions of the hippocampus in mammals. The results of this research can help explain the selectivity of hippocampal lesions during ischemic injuries and neurodegenerative diseases.

Keywords: NADH production, brain, mitochondria, electron transport chain (ETC), hypoxia, cortex, cerebellum, hippocampus, brainstem.

1. INTRODUCTION

Eukaryotic cells harvest energy mainly by respiration, i.e. with the consumption of oxygen molecules. During dissimilation, nutrients are cleaved to simpler compounds, whereafter they enter the tricarboxylic acid cycle (TAC) in mitochondria. TAC is a cycle of 12 chemical reactions resulting in critical signalling compounds formation comprising nicotinamide dinucleotide coenzyme (NADH). NADH then becomes a substrate for electron transport chain (ETC) where cellular respiration occurs[1].

ETC is composed of enzymatic complexes that carry out the oxidative phosphorylation reactions. NADH enters complex I and electrons are then passed along the ETC, creating a counterflow to protons located in the intermembrane space of the mitochondria. That process forms the mitochondrial membrane potential ($\Delta\Psi m$), which is necessary for the normal adenosine triphosphate (ATP) synthesis. ATP is a molecule with macroergic bonds and the primary source of energy for most cellular processes. Thus, the cell vital activity depends on the balanced functioning of the energy metabolism elements (TAC, ETC)[2]. Vice versa, any of these elements dysfunction can lead to hypoxia, degenerative processes and cell death[3]. Evaluation of NADH can provide useful information about mitochondrial viability and safety of the whole metabolic pathway.

NADH (NAD+, NADH) is a coenzyme that is present in all living cells and together with nicotinamide dinucleotide phosphate (NADPH, NADP+) is part of the dehydrogenase enzymes class involved in redox reactions of energy metabolism. Determination of NADH and NADPH by the intensity of their autofluorescence is commonly used. Detection of endogenic molecules by the parameters of their fluorescence allows one to determine even minimal quantities of the substances[4,5]. The method used in the study is based on the property of reduced forms of nicotinamide coenzymes NAD(P)H to fluorescence when excited by a light with a wavelength of 375 nm, giving a maximum of emission at 480 nm[6]. NAD(P) evaluation has been widely used to study the effects of hypoxia and ischemia in various tissues [7, 8, 9].

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Albeit similar pathological processes during mitochondrial and cellular respiration impairment are observed in most diseases, cell respiration failure and related pathological conditions play a pivotal role in diseases of the central nervous system (CNS)[10]. It is shown that neurons and neuroglia have a low resistance to hypoxia and rapidly die during mitochondria dysfunction. Thus, hypoxic-ischemic damage is a critical mechanism in the pathogenesis of stroke[11], as well as mitochondrial dysfunction and associated oxidative stress in neurodegenerative diseases[12]. Despite intensive research conducted in this area, many mechanisms of degenerative conditions development remain unclear.

In the nervous tissue, the physiological rate of NAD(P)H formation as well as the activity of mitochondria vary[13]. Cells of different brain regions have different values of metabolic activity[14]. The least stable are hippocampal neurons (CA1 field cells), black substance, blue spot cells, etc. Later other parts of the brain (including neocortex) are involved in the pathology[15]. That leads to the need for a differentiated assessment of cellular respiration activity in various areas of the CNS accounting the individual tissue characteristic of the region.

Thus, this work aimed to compare the NAD(P)H production rate in the tissues of various brain regions (cortex, cerebellum, hippocampus, brainstem) assessed by the parameters of its autofluorescence.

2. MATERIALS AND METHODS

All experiments were conducted according to the standards of ethical treatment of animals[16] and were approved by the Ethics Committee of the Orel State University named after I. S. Turgenev (Protocol No. 18 of 21.02.2020). Brain tissues of male Wistar rats aged 10 to 12 weeks (n=2) were used in this research. The animals were obtained from "Nursery for laboratory animals" of Institute of Bioorganic Chemistry and were kept in standard conditions (cycle 12 hours/light/dark). Euthanasia was performed by cervical dislocation. After decapitation and brain extraction, we cut acute brain slices of the cortex, cerebellum, hippocampus and brainstem (Fig.1). Six tissue slices from each analysed brain area were performed.

To measure mitochondrial NADH an experimental setup with a BDS-SM-375-FBC-101 laser source (Becker&Hickl, Germany) was assembled to excite NADH fluorescence at a wavelength of 375 nm (Fig. 2). In the fluorescent imaging channel, the back-reflected radiation of the source passes through the light filters and is registered by a highly sensitive CCD camera DCC 3260C (Thorlabs etc, USA). The field of view of the camera is a rectangular area of about 1 mm^2 on the sample.

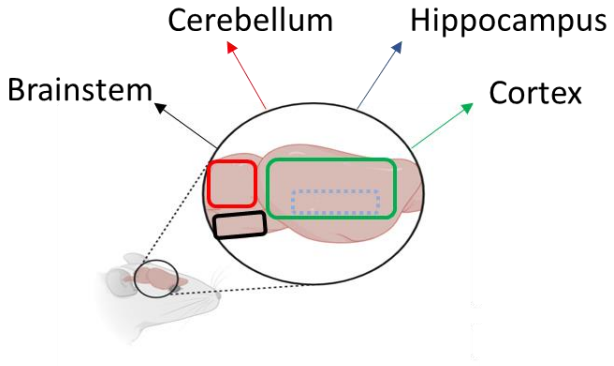

Figure 1. Rat brain regions evaluated in the study

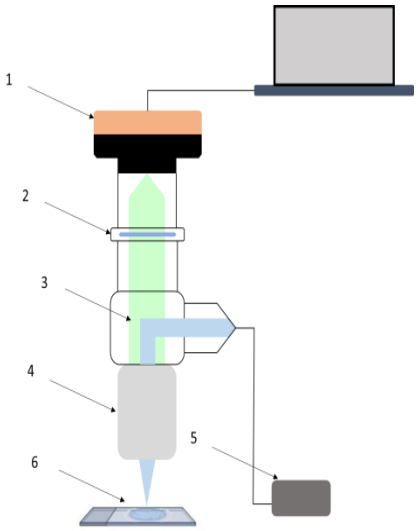

Figure 2. Layout of the used imaging setup: 1 – 340m-USB CCD (Thorlabs, USA) camera; 2 – lens f=200 mm; 3 – dichroic filter MD416 (Thorlabs, USA); 4 – collimator CVH100-COL (Thorlabs, USA); 5 – laser BDS-SM-375-FBC-101 (B&H, Germany); 6 – tissue sample.

In a series of measurements, the basal fluorescence level, fluorescence intensity after addition of FCCP and NaCN were analysed. The interval between frames during the imaging procedure was 2 s. The received data were processed with the OriginPro (2015) program.

Analysed brain tissues were fixed in a 10% buffered formalin solution to control the viability of tissues and the degree of morphological changes. Histological processing was performed according to the standard method with subsequent filling in paraffin. The HM 340E (Thermo Scientific) rotary microtome was used to make 4-mm-thick slices, which then were stained with H&E. Pathomorphological changes in the tissue were microscopically evaluated.

3. RESULTS AND DISCUSSION

In the brain slices, FCCP decreased the registered fluorescence intensity to minimum values. That is the result of the oxidative phosphorylation uncoupling by destroying the proton gradient on the inner mitochondrial membrane and causing a decrease of mitochondrial NADH by its intensive oxidation to a minimal level. Addition NaCN to the imaging chamber increased the intensity of NADH fluorescence which reached values above the initial basal level. Since cyanides are a specific inhibitor of ETC, NaCN prevents NADH from oxidation and increase its abundance in the mitochondria. The described effects of FCCP and NaCN was observed in all the examined parts of the brain.

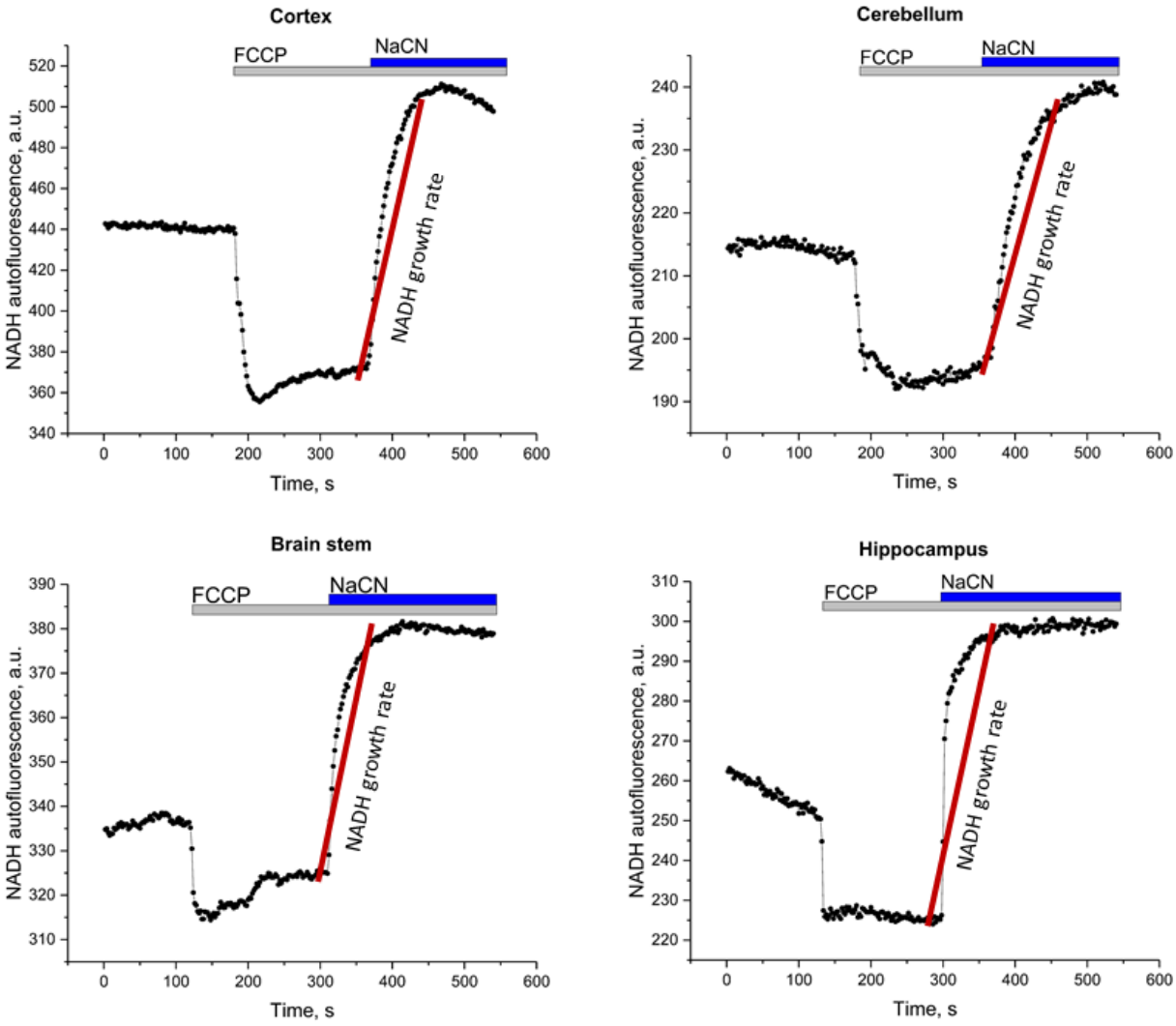

Figure 3. Examples of change of the registered fluorescence intensity in selected brain regions of rat**.**

The rate of increase in the fluorescence intensity of NAD(P)H after NaCN was added, corresponds to the NADH growth rate. It characterises the effectiveness of the TAC and ETC activity. Examples of the fluorescence intensity change in selected brain regions during the implemented experimental procedure are shown in Figure 3. Data analysis has shown that hippocampus has a higher rate of NADH production (2.43±0.61 a.u./sec) in comparison to other parts of the brain (cerebellum - 1.35±0.22 a.u./sec, the cortex - 1.67 ± 0.66 a.u./sec, brain stem - 1.11±0.54 a.u./sec.). Statistically significant differences were obtained in the hippocampus in comparison with the cerebellum ($p<=0.01$) and the brainstem ($p<=0.001$). Statistical analysis was performed using the Mann-Whitney U test (Figure 4).

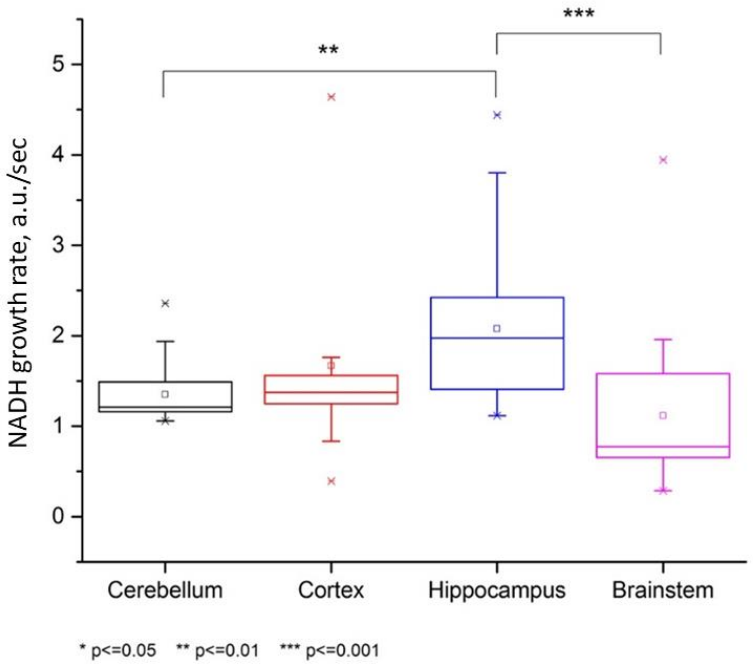

Figure 4. Statistical analysis of NADH growth rate in different brain regions with the Mann-Whitney U test.

Histological examination of tissues showed that the brain vessels were mostly full-blooded, edema and expansion of perivascular spaces were visualised. The histoarchitectonics of all the studied brain regions was preserved. Most neurons were normochromic, round-shaped, several with swelling, a small number of neurons were of cell-shades manner. At the same time, few cells with eosinophilic (oxyphilic) degeneration appeared. Morphological examination shows moderate hypoxic changes. The described pathomorphological changes may correspond to the stages of post-traumatic state and functional activity restoration.

According to the results, the highest intensity of mitochondrial activity was observed in the hippocampal tissues, which is probably an indication of the importance and complexity of the cognitive functions performed. Maintaining the redox state is necessary for normal cell activity since numerous metabolic, signalling and transcription processes are regulated by the cellular redox[17]. We analysed the intensity of NAD(P)H production to identify brain regions that may be more susceptible to hypoxia and other pathological conditions associated with mitochondrial dysfunction (for example, neurodegenerative diseases). Pathomorphological control of the analysed tissues allowed us to conclude the validity of the data obtained and assess the degree of hypoxic and degenerative changes in the tissue.

The data obtained about the levels of NADH fluorescence in various regions of the brain is consistent with other studies[18] showing a higher level of energy production in the hippocampus[19]. Besides, it should be noted that mitochondria are more active in hippocampal neurons compared to astrocytes of this region[17].

It should also be mentioned that the study of Braidy, *et al* [20] was aimed at measuring autofluorescence in rats of different ages (from the youngest to the oldest), which allowed them to establish a correlation of NADH level with age and compare the bioenergetics of brain regions in different periods of ontogenesis. Despite the differences in the use of the amount of the main ETC inhibitor (authors used KCN 1-2 mM, in our work – NaCN 5 mM), there are similarities in statistical differences in the hippocampus with the cerebellum ($p<=0.01$) and the brainstem ($p<=0.001$).

4. CONCLUSIONS

In this study, using in-house built imaging system, we have analysed the parameters of the dynamics of NAD(P)H endogeneous fluorescence in acute slices of the rat brain and revealed a higher NAD(P)H growth rate in hippocampal tissue. The finding reflects the high intensity of mitochondrial metabolic processes in the type of tissue. Initial stressed level of bioenergetic processes in the hippocampus can be a factor of a higher risk of disruption of the bioenergetics during hypoxia and neurodegeneration.

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